1. Synaptic rearrangements in relationship with the forelimb functional map

To more precisely determine the location in the motor cortex of our *in vivo* images, intracortical microstimulation was used to map forelimb movements evoked at these sites. In total, 14 mice were successfully mapped in this study. This included 5 and 3 young mice imaged over 4 and 8 days respectively, and 4 and 2 adult mice imaged over 4 and 8 days respectively. The average motor response map and 3 representative examples with imaging sites marked are shown in Supplementary Fig. 2. We found that images of all 14 mice were located in the functional motor cortex. Imaging locations relative to maps of the forelimb movement representation could be classified into three categories: 1) the imaging region fell completely within forelimb map (n=5); 2) the imaging region was located on the border of the forelimb map (n=6); and 3) the imaging region was located outside the forelimb map, but within 300 μm of its border (n=3). Given the fact that dendritic arbors of layer V neurons are at least 300 μm across, dendritic branches imaged in the last category may still have some involvement in forelimb movements. In addition, when we imaged at a distance from the forelimb region (>500 μm posterior to the forelimb border), we found no change in synaptic dynamics following 4 days of training (n=4, P>0.2 comparing to controls, Fig. 2a, b). All together, these results suggest that synaptic rearrangements are regional specificity. However, whether there is a correlation between spine dynamics and their exact position within the forelimb map remains to be explored.

Earlier studies have shown that motor map reorganization occurs slowly, not during initial learning, but rather after the motor skill has been acquired[32]. High spine dynamics observed at the border of forelimb maps during early learning acquisition in this study suggests that synapse remodeling may precede and provide a structural basis for functional map remodeling. It will be ideal to follow the synapse dynamics and the functional reorganization in the same mouse over time. Unfortunately,

intracortical microsctimulation experiments require penetration of electrodes into the cortex and mice cannot be imaged for structural changes afterwards. The recent development using non-intrusive photoactivation of channelrhodopsin-2 mice to map the functional motor cortex could provide a potential approach to further address this question[33,34].

2. Estimation of numbers of neurons imaged

YFP-H mice have a high density labeling, which makes it impossible to trace individual layer V neurons. However, we chose this mouse line for the current study because its high labeling density throughout the motor cortex guaranteed dendritic labeling through the tiny thin-skull region (~300 μm in diameter). To estimate how many cells were sampled on average per mouse, the skull above the imaged region was removed after the final image and a small metal pin soaked with 1% DiI solution was used to mark the surface of the imaged region. Brains were dissected out and post-fixed in 4% paraformaldehyde overnight and coronal slices (200 μm thick) at the level of DiI-labeled brain regions were cut with a vibratome and imaged with a 2-photon microscope. 100 μm image stacks were maximum projected onto a single plane and numbers of YFP+ cells were counted over a 200 μm wide column throughout layer V. These numbers were roughly half of the neuron numbers in our imaged window (200μm × 200 μm × layer V depth). In this way, we estimated that ~20-80 cells were sampled for each mouse.

3. Effects of motor learning on dendritic branching

Previous studies have shown that extended motor skill learning (14-16 days) increases dendritic length and branching complexity of pyramidal cells in the contralateral motor cortex of rats[35-37]. Due to the high degree of YFP-H line labeling, we were not able to trace back the whole dendritic tree of the

same neuron to analyze dendritic complexity. In this study, we examined superficial dendritic branches instead and did not find obvious addition or removal of dendritic branches with imaging intervals up to 16 days. This observation is consistent with an earlier study carried out on GFP-M line – another line with sparse labeled layer V pyramidal neurons[38]. However, tips of terminal dendrites exhibit small extension and retraction over time, with 3.0±0.4 μm extension/retraction over 16 days in control mice (39 tips analyzed from 3 mice). No significant difference in tip dynamics was observed between trained and control groups over 16 days (2.8±0.3 μm, 63 tips analyzed from 4 trained mice, $P>0.5$).

4. Motor activity alone induces no increase in spine formation

In this study, we tried to obtain activity controls for both short and long periods of training. However, because most activity controls gave up reaching after 6-8 days of training, it has been hard to obtain enough control data for long-term activity to enable a statistical evaluation. Among a total 8 activity control mice that were trained for more than 4 days, only 2 of them continued to reach >20 times/day at 8 days. We imaged both mice and found 7.0% spine formation in both cases, comparable to the general controls during the same period of training (7.0±0.9%). One of these mice continued to reach until day 16. It was imaged and found to have 8.2% spine formation over 16 days. This number is also comparable with the general control mice (8.7±1.3%). These data, together with the low rate for formation of new spines seen during retraining adult mice to an 8-day reaching task (4 mice), suggest that activity alone is unlikely to be responsible for appreciable spine changes as measured in this study. Finally, our results are also consistent with earlier mapping studies that show expansion of movement representations in rats occurs only in response to motor skill learning, but not repetitive motor activities[32].

5. Fate of new spines formed during different learning phases

To determine the survival rate of new spines formed during different learning phases, we imaged mice under three scenarios (Supplementary Fig. 3). 1) Early learning acquisition phase: mice were imaged the day before training and immediately after day 1 and day 5 of training. 2) Late learning acquisition phase: mice were imaged after day 3, 4 and 8 of training. 3) Learning maintenance phase: mice were imaged after day 11, 12 and 16 of training. Mice showed significant behavioral improvement during learning acquisition, but not learning maintenance.

We found that 39.7±1.9% of the new spines formed on the first day of training persisted till day 5 (693 spines, 4 mice). This number was significantly higher than control mice imaged over the same intervals (26.4±3.5%, 658 spines, 4 mice, $P<0.005$), in agreement with our current conclusion that later learning selectively stabilizes earlier learning induced new spines. We also found that enhanced spine formation was closely associated with behavioral improvement. A small but still significantly higher spine formation was observed on training day 4 ($P<0.05$), when a small improvement of reaching successes was observed. Spine formation was comparable to control mice, after reaching success rates reached the plateau (training day 12, $P>0.6$). In addition, 32.5±1.7% spines formed on day 4 of training survived till day 8 ($P<0.05$ comparing to control); while 29.8±2.4% spines formed on day 12 of training survived till day 16 ($P>0.1$ comparing to control). These results indicate that initial learning acquisition plays a more important role in both synaptogenesis and rewiring of neuronal circuitry.

6. Effects of cross-training on spine sizes

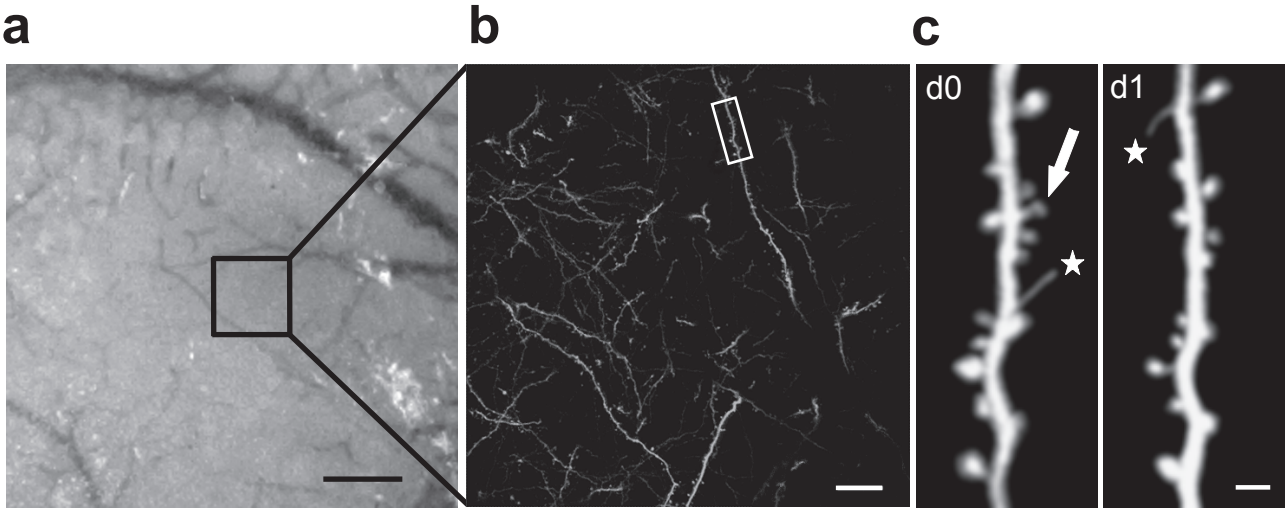
It is known that the spine size is closely correlated to synaptic strength[39]; and changes in synaptic connectivity, such as LTP and LTD, have been shown to lead to spine enlargement or shrinkage[40,41]. Therefore, we followed the size of individual spines that appeared during motor learning and persisted

into adulthood. ImageJ was used to measure the diameter of the spine head and the nearby dendritic shaft[42]. Because imaging and animal conditions varied over time, the ratio of the spine head diameter to the adjacent dendritic shaft diameter was used as the calibrated spine head diameter. The change in the spine head diameter was defined as the difference in the calibrated spine head diameter between two views. We found new spines formed during adolescent reaching tasks, if they still persisted, maintained relatively stable diameters during 4 days on the capellini handling task in adulthood (29 spines, $P>0.08$, paired *t*-test), further suggesting the stability of matured synapses. This result is consistent with the behavioral data showing that, in pretrained adults, skilled reaching behavior is maintained following 4-8 days of training on the capellini task. It further supports the idea that different motor behaviors are stored using different sets of synapses in the brain. However, due to technical limitations, our spine size analysis over chronic 2-photon imaging provides only limited insights on synaptic strength. Changes in synaptic strength will have to be addressed by other direct recording techniques in future studies.

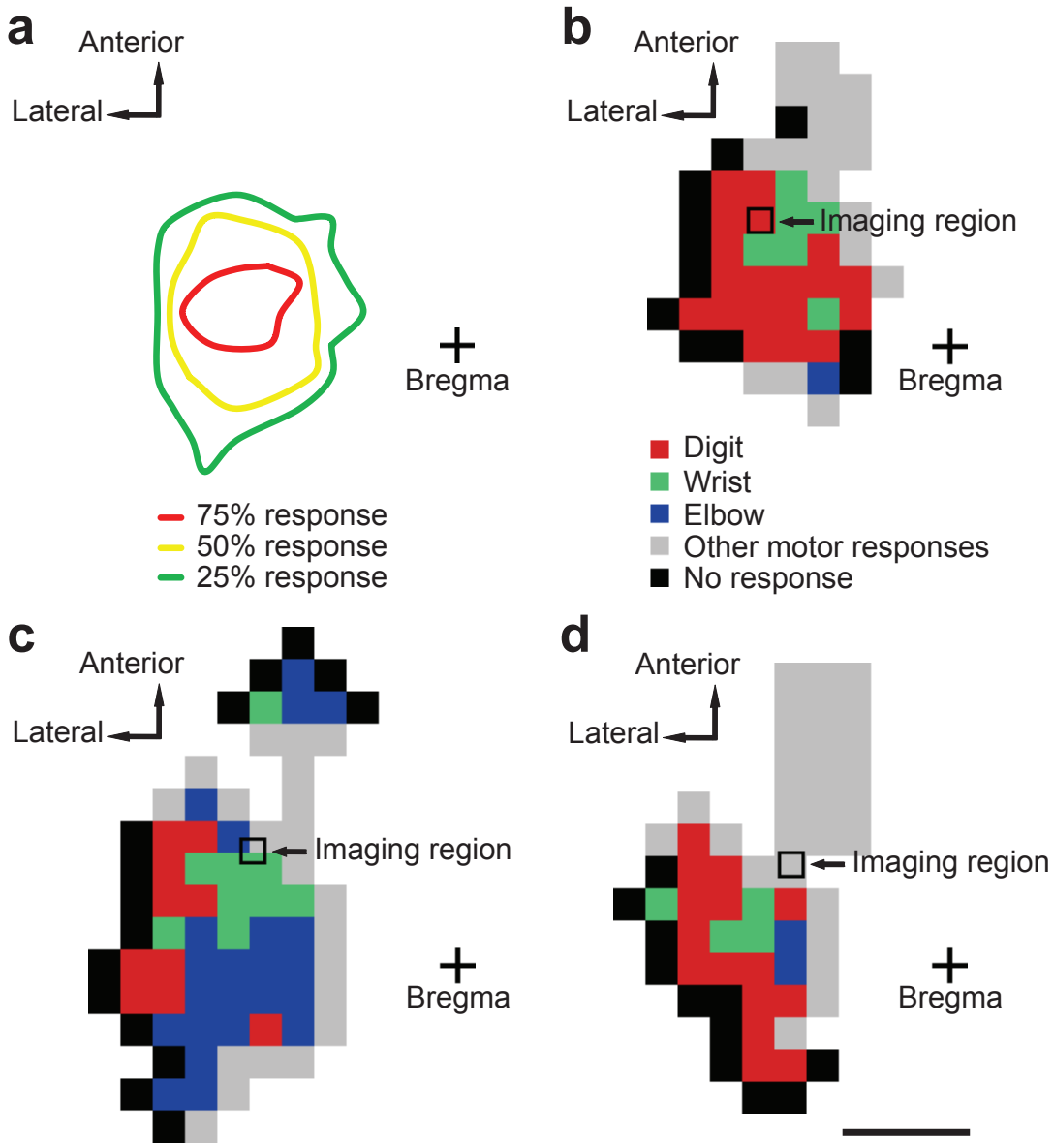
References for Supplementary Information

32. Kleim, J.A. *et al.* Cortical synaptogenesis and motor map reorganization occur during late, but not early, phase of motor skill learning. *J Neurosci* **24,** 628-633 (2004).
33. Ayling, O.G., Harrison, T.C., Boyd, J.D., Goroshkov, A. & Murphy, T.H. Automated light-based mapping of motor cortex by photoactivation of channelrhodopsin-2 transgenic mice. *Nature methods* **6,** 219-224 (2009).
34. Hira, R. *et al.* Transcranial optogenetic stimulation for functional mapping of the motor cortex. *Journal of neuroscience methods* **179,** 258-263 (2009).
35. Greenough, W.T., Larson, J.R. & Withers, G.S. Effects of unilateral and bilateral training in a reaching task on dendritic branching of neurons in the rat motor-sensory forelimb cortex. *Behavioral and neural biology* **44,** 301-314 (1985).
36. Withers, G.S. & Greenough, W.T. Reach training selectively alters dendritic branching in subpopulations of layer II-III pyramids in rat motor-somatosensory forelimb cortex. *Neuropsychologia* **27,** 61-69 (1989).
37. Kolb, B., Cioe, J. & Comeau, W. Contrasting effects of motor and visual spatial learning tasks on dendritic arborization and spine density in rats. *Neurobiology of learning and memory* **90,** 295-300 (2008).
38. Trachtenberg, J.T. *et al.* Long-term in vivo imaging of experience-dependent synaptic plasticity in adult cortex. *Nature* **420,** 788-794 (2002).

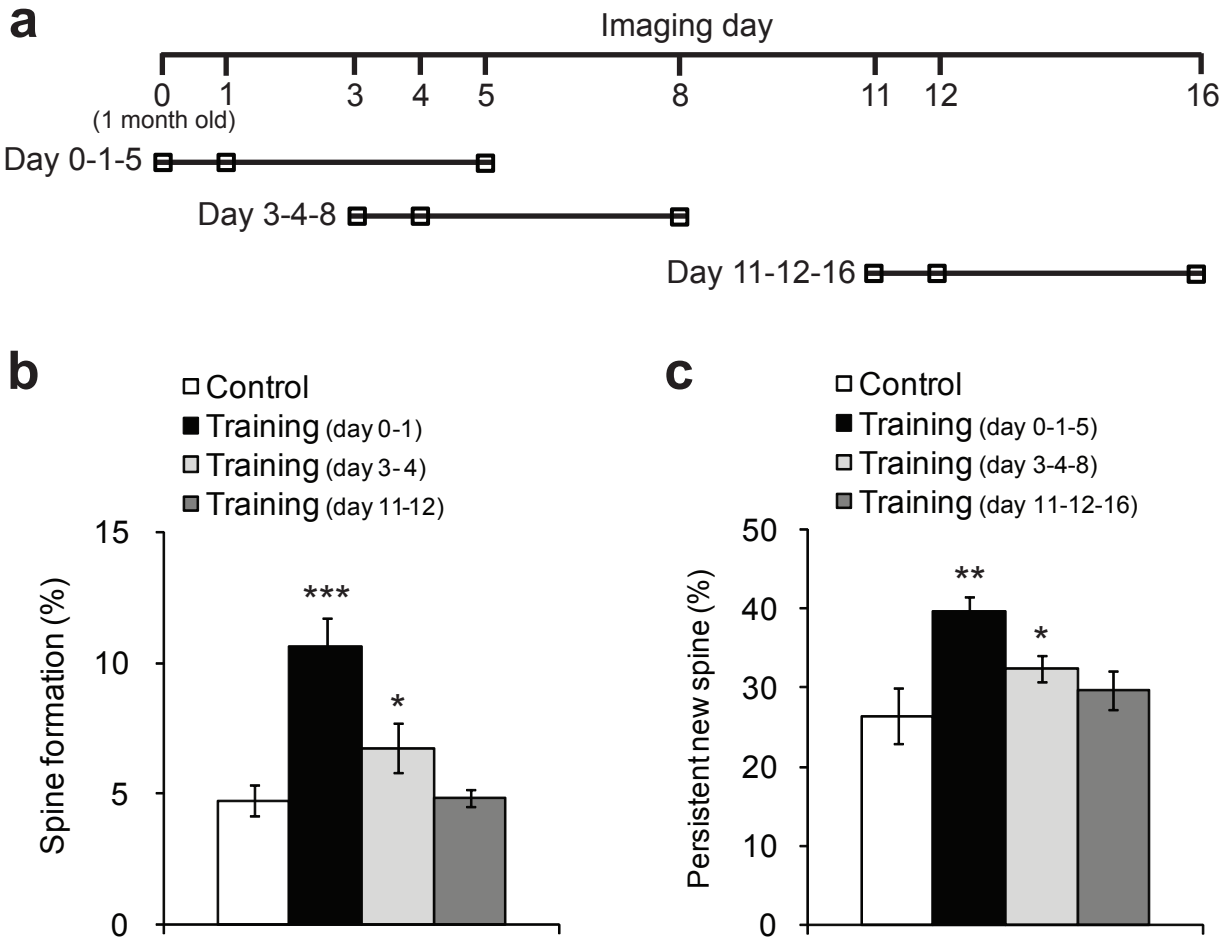
39. Kasai, H., Matsuzaki, M., Noguchi, J., Yasumatsu, N. & Nakahara, H. Structure-stability-function relationships of dendritic spines. *Trends in neurosciences* **26,** 360-368 (2003).
40. Matsuzaki, M., Honkura, N., Ellis-Davies, G.C. & Kasai, H. Structural basis of long-term potentiation in single dendritic spines. *Nature* **429,** 761-766 (2004).
41. Zhou, Q., Homma, K.J. & Poo, M.M. Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. *Neuron* **44,** 749-757 (2004).
42. Zuo, Y., Lin, A., Chang, P. & Gan, W.B. Development of long-term dendritic spine stability in diverse regions of cerebral cortex. *Neuron* **46,** 181-189 (2005).



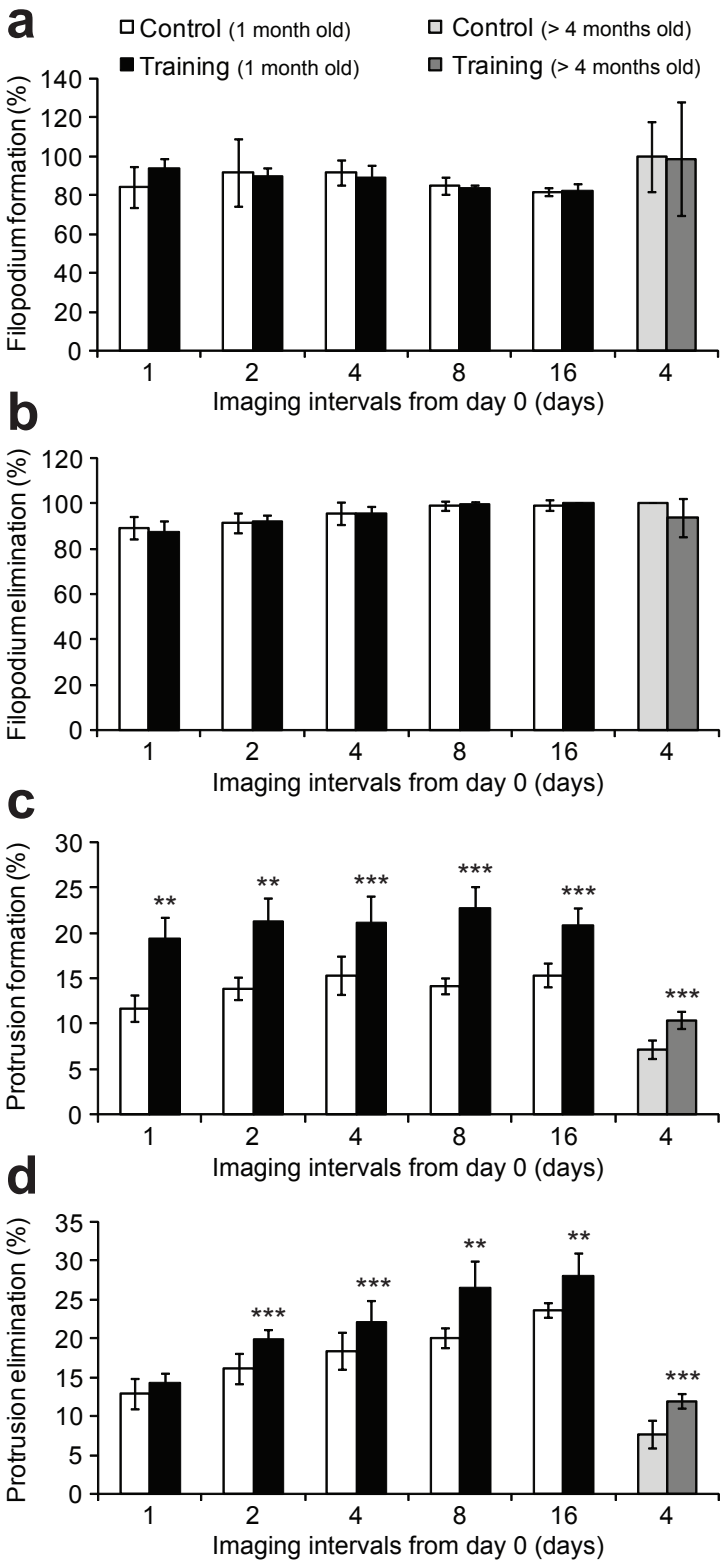
Supplementary Figure 1: Transcranial two-photon imaging of the primary motor cortex from a one-month-old mouse showing dynamics of dendritic spines over one day. **a**, An image obtained from a CCD camera of the vasculature underneath the thinned skull. The black square indicates the region where subsequent two-photon images were obtained. **b**, A low-magnification two-dimensional projection from a three-dimensional stack of dendritic branches and axons in the motor cortex. A higher-magnification view of a dendritic segment in the white box is shown in the left panel of (**c**). **c**, Two images of the same dendritic branch obtained one day apart reveal spine elimination (the arrow) and filopodia (stars) in a control mouse. Scale bars: 200 μm (**a**), 20 μm (**b**) and 2 μm (**c**).



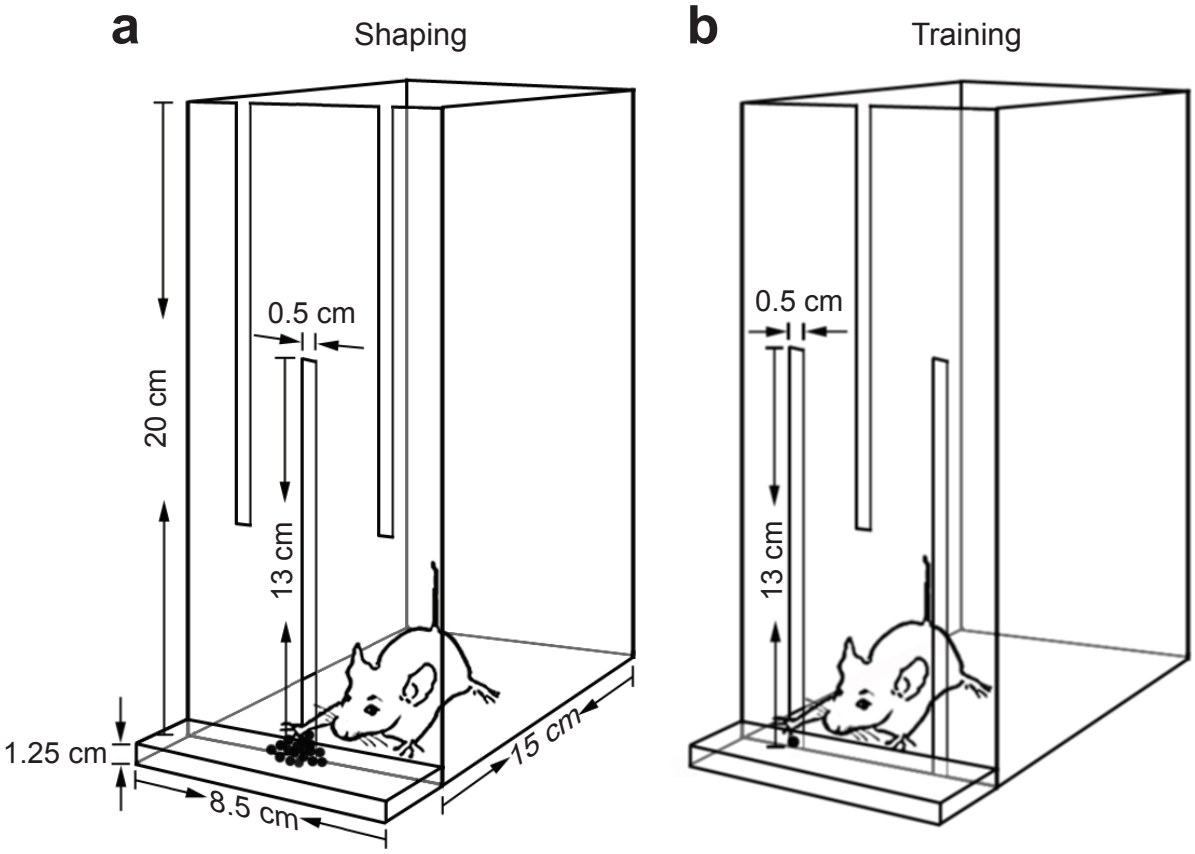
Supplementary Figure 2: Locations of imaged regions in relation to the forelimb functional map. **a**, An average forelimb response map from 14 mice. Contour lines outline regions in which 75%, 50%, 25% of animals have microstimulation evoked forelimb movements. **b-d**, Examples showing three typical locations of the imaging region relative to the forelimb response map. **b**, The imaging region falls completely into the forelimb map. **c**, The imaging region falls on the border of the forelimb map. **d**, The imaging region is located outside the forelimb map, but within 300 μm of the forelimb border. Regions with forelimb movements are shown in color. Regions in which other movements are evoked (such as hindlimb, trunk, whisker etc) are indicated in grey. Regions without any motor responses are indicated in black. The black square indicates the site of *in vivo* imaging, and the cross indicates the location of bregma. Scale bar: 1 mm.



Supplementary Figure 3: **New spines formed during learning acquisition, but not learning maintenance, have higher survival rates than controls.** **a**, Timeline of experiments, showing the imaging days. **b**, Percentages of spines formed over one day in control, and on training day 1, 4 and 12. **c**, Survival rates of new spines in all the categories in (**b**) over 4 days (mean±s.d., **P*<0.05, ***P*<0.01 and ****P*<0.001).



Supplementary Figure 4: Dynamics of filopodia and total dendritic protrusions in various control and training mice. a, b, Percentages of filopodia formed (**a**) and eliminated (**b**) under various control and training conditions. **c, d,** Percentages of total dendritic protrusions (filopodia plus spines) formed (**c**) and eliminated (**d**) under various control and training conditions. Data are presented as mean±s.d., ***P*<0.01, ****P*<0.001.



Supplementary Figure 5: A cartoon drawing of the animal chamber used during shaping and training. Dimensions are indicated on the picture. **a**, During 'shaping', seeds are centered in front of the middle slit and mice use both paws to reach for them. **b**, The chamber is flipped upside-down during training. Seeds are placed individually only at the side slit of the preferred limb (in this example, the right limb).

Supplementary Table S1

Percentages of spines eliminated and formed over various intervals under different experimental conditions. Control mice (general controls) are littermates that undergo the same food restriction and are handled similarly as trained mice, but without either shaping or training. The motor cortex contralateral to the trained limb is imaged in all training categories, unless otherwise stated. Data are presented as mean ± s.d., $^{**}P<0.01$, $^{***}P<0.001$ compared to controls (shown in red).

Imaging intervals	Experimental conditions	Spine formation (%)	Spine elimination (%)	Total number of spines	Total number of mice
Young mice (1 month old)					
1 day	Control	4.7 ± 0.6	5.7 ± 0.9	1335	8
	Shaping control[#]	4.1 ± 0.6	5.1 ± 1.2	542	4
	Activity control[##]	4.8 ± 0.7	5.6 ± 0.8	535	4
	Training[$]	***10.6 ± 1.1	5.6 ± 0.9	559	4
2 days	Control	5.3 ± 0.6	7.7 ± 1.6	1720	11
	Training	**13.7 ± 3.1	***11.8 ± 1.5	915	5
4 days	Control	6.0 ± 1.2	9.0 ± 1.0	2510	18
	Training	***13.3 ± 2.3	***13.7 ± 2.0	3881	25
	Training (ipsilateral motor cortex)	5.2 ± 1.1	8.7 ± 1.0	588	4
	Training (sensory cortex)	6.3 ± 0.7	9.2 ± 1.2	574	4
	Fail to learn	6.1 ± 1.7	8.7 ± 1.3	640	5
8 days	Control	7.0 ± 0.9	12.2 ± 1.0	864	5
	Training	***15.5 ± 1.7	***17.7 ± 2.1	1077	5
16 days	Control	8.7 ± 1.3	16.1 ± 0.8	1233	8
	Training	***13.6 ± 2.0	**19.7 ± 2.7	1454	8
Adult mice (> 4 months old)					
4 days	Control	3.7 ± 0.8	4.0 ± 1.2	743	6
	Training (reaching task)	***7.9 ± 1.4	***9.5 ± 0.8	806	5
	Training (capellini task)	***7.8 ± 0.6	***8.2 ± 0.8	552	5
	Retraining (reaching task)	4.4 ± 0.9	4.7 ± 0.7	601	5
	Retraining (capellini task)	***8.7 ± 0.5	***9.0 ± 0.7	620	5
8 days	Control	4.9 ± 0.9	5.0 ± 1.7	490	4
	Training (reaching task)	**8.7 ± 1.1	**10.5 ± 2.0	514	4
	Retraining (reaching task)	4.9 ± 0.9	5.2 ± 0.8	459	4

[#] Mice go through shaping, but are not trained to reach. Shaping controls are used to determine whether the shaping period and/or the experience of the training environment have any effects on spine dynamics.
[##] Mice go through shaping, and are trained to reach for the food pellet far away to grasp. Activity controls have similar amounts of forelimb activity as trained mice, but never develop skillful movements.
[$] Trained mice with > 10 successes on day one of training.